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Chapter 12: Conventional Diagnostic Methods

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12.1. Introduction

Active tuberculosis (TB) is diagnosed by detecting *Mycobacterium tuberculosis* complex bacilli in specimens from the respiratory tract (pulmonary TB) or in specimens from other bodily sites (extrapulmonary TB). Although many new (molecular) diagnostic methods have been developed, acid fast bacilli (AFB) smear microscopy and culture on Löwenstein-Jensen medium are still the “gold standards” for the diagnosis of active TB and, especially in low-resource countries, the only methods available for confirming TB in patients with a clinical presumption of active disease. AFB smear microscopy is rapid and inexpensive and thus is a very useful method to identify highly contagious patients. Culture is used to detect cases with low mycobacterial loads and is also requested in cases at risk of drug-resistant TB for drug susceptibility testing, or in cases where disease due to another member of the *Mycobacterium* genus is suspected. AFB smear microscopy and culture can also be used to monitor the effectiveness of treatment and can help to determine when a patient is less likely to be infectious. Two manuals are recommended for the laboratory diagnosis of TB (Kent 1985, Master 1992). Good reviews on this issue are also available on the internet at http://www.phppo.cdc.gov/dls/ila/TB_Toolbox.aspx.

The authors of this chapter wish to emphasize that accurate case detection is the rate-limiting step in TB control (Perkins 2002). While as many as two thirds of sputum smear-positive cases probably remain undetected worldwide, the efforts to control the disease have focused more on curing TB cases than on detecting them. (Gupta 2004, Dye 2003). Even though the laboratory is essential for the diagnosis and control of TB, it does not receive enough attention in developing countries, where AFB sputum microscopy is often the only available method to diagnose TB. Sputum microscopy lacks sensitivity, especially in children and in people living with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS). Its usefulness depends largely on the quality of the sputum specimen and the performance quality of the laboratory. Considerable efforts have been made to improve the sensitivity of sputum smear microscopy (Steingart 2006) and special emphasis will be given in this chapter to these efforts.

Culturing of *M. tuberculosis* has become increasingly important in the last decades, in particular because of the need for drug resistance testing. As most laboratories in

low-resource countries have no access to culturing mycobacteria, alternative simple culturing methods will be discussed, as well as the value of alternative culture media such as blood agar, which is more readily available in most laboratory settings than the traditional egg-based media used for mycobacterial isolation. Although no multicenter studies have been published to show their efficiency for cultivating mycobacteria, we think that these alternatives should be presented in this chapter, because they may be particularly useful in settings where standard procedures simply cannot be performed due to the absence of laboratory equipment or reagents. In addition, we will discuss the utility of adenosine deaminase activity for the diagnosis of extrapulmonary TB: tuberculous meningitis, tuberculous pleural effusions, and tuberculous peritonitis. These presentations of TB are difficult to diagnose by smear staining and culture, and for this reason, they probably remain underdiagnosed in resource-poor countries.

12.2. Specimen handling

12.2.1. Specimens

The successful isolation of the pathogen requires that the best specimen be properly collected, promptly transported and carefully processed. Many different types of clinical specimens may be obtained for the microbiological diagnosis. If pulmonary TB is suspected, specimens originating from the respiratory tract should be collected, i.e. sputum, induced sputum, bronchoalveolar lavage or a lung biopsy. For the diagnosis of pulmonary TB, three first-morning sputum specimens (not saliva) obtained after a deep, productive cough on non-consecutive days are usually recommended. Several studies have shown, however, that the value of the third sputum is negligible for the diagnosis of TB, as virtually all cases are identified from the first and/or the second specimen (Yassin 2003, Nelson 1998, Dorransoro 2000, Finch 1997). Before processing, sputum specimens must be classified at the laboratory with regard to their quality, i.e. bloody, purulent, mucopurulent, saliva (see also section 12.2.2).

In patients who cannot produce it spontaneously, the sputum can be induced by inhalation of hypertonic saline solution. Otherwise, the specimen can be collected from bronchoscopy. This intervention usually provokes cough and post-bronchoscopy expectorated sputum specimens should be collected because they often provide satisfactory microorganism yields (Sarkar 1980, de Gracia 1988). Some studies suggest that a single induced sputum specimen is equally effective as bronchoscopy for diagnosing pulmonary TB (Anderson 1995, Conde 2000). A recent study demonstrated that the most cost-effective strategy is to perform three

induced sputum tests without bronchoscopy (MacWilliams 2002). On the contrary, transbronchial biopsy specimens contribute little to the bacteriological diagnosis of TB (Stenson 1983, Chan 1992). “Fasting” gastric aspirates is the specimen of choice in the case of young children who cannot cough up phlegm. Gastric lavage fluid must be neutralized with sodium carbonate immediately after collection (100 mg per 5-10 mL specimen).

Specimens to be collected for the diagnosis of extrapulmonary disease depend on the site of the disease. The most common specimens received in the laboratory are biopsies, aspirates, pus, urine, and normally sterile body fluids, including cerebrospinal fluid, synovial, pleural, pericardial, and peritoneal liquid. Stool can be collected when intestinal TB is suspected and also in the case of suspected *Mycobacterium avium* infection in AIDS patients. Whole blood and/or bone marrow specimens are collected only if disseminated TB is suspected, mainly in patients with an underlying severe immunosuppressive condition such as AIDS. Bone biopsies are the specimen of choice when skeletal TB is suspected. In general, AFB smear microscopy from body fluids is rarely positive and the whole sediment from concentrated specimens should rather be cultured. In tuberculous pleural effusions, the diagnostic value of the pleural biopsy is much higher than that of the fluid and, therefore, is the specimen of choice for the diagnosis (Escudero 1990, Valdez 1998). The diagnosis of peritoneal and pericardial TB is difficult and usually requires invasive procedures such as laparoscopy and biopsy.

Specimens should be collected in sterile, leak-proof containers and labeled with the patient’s name and/or identification number before anti-tuberculosis chemotherapy is started. Induced sputum specimens should be labeled as such because they resemble saliva and may be disregarded at the laboratory. Specimens must be collected aseptically in order to minimize contamination with other bacteria.

Blood and other specimens prone to coagulate, including bone marrow, synovial, pleural, pericardial and peritoneal fluids, should be collected in tubes containing sulfated polysaccharides or heparin. Sulfated polysaccharides are the preferred anticoagulants as they enhance the growth of mycobacteria. Heparinized specimens are also satisfactory, but specimens collected in ethylenediaminetetraacetic acid (EDTA) are unacceptable as even trace amounts of this substance inhibit mycobacterial growth. Lymph nodes, skin lesion material, and tissue biopsy specimens should come without preservatives or fixatives and should not be immersed in saline or any fluid. Once in the laboratory, tissue specimens are homogenized in a sterile tissue grinder with a small amount of sterile saline solution before AFB smear staining or culture. Abscess contents or aspirated fluids can be collected in a syringe. If renal TB is suspected, the specimen of choice is the first-morning urine,

at least 50 mL, obtained by catheterization or from the midstream clean catch on three consecutive days. Urine specimens should be submitted to a decontamination step for mycobacteria prior to cultivation.

12.2.2. Specimen transport

Diagnostic specimens (sputum, blood, tissue) and mycobacterial cultures can be mailed, but transportation of dangerous or infectious goods is regulated by law in many countries. Specific shipping instructions can be found in the guide by Kent (Kent 1985). Shipments by private couriers are regulated by the International Air Transportation Association (IATA), and its shipping guidelines for infectious substances can be found on the internet at <http://www.iata.org/training/courses/tcgp22.htm>. The guidelines released by the WHO in 2007 are available at http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2/en/index.html. Mailing instructions for specimens and cultures are also available at the National Jewish Hospital site: http://www.njc.org/pdf/2005_shipping_instructions.doc.

Specimens should be transported rapidly to the laboratory to avoid overgrowth by other microorganisms. This is particularly true for specimens from non-sterile sites, such as sputum. When the transport or the processing is delayed, specimens should be stored for not more than five days at 4°C until transported or presented for bacteriological processing. The cetylpyridinium chloride (CPC) method is widely used for the transport of sputum specimens (Smithwick 1975). CPC eliminates the associated flora in sputum specimens and treated specimens should not be submitted to further decontamination prior to cultivation. The detection of AFB with Ziehl-Neelsen staining can be significantly reduced in specimens preserved by this method (Selvakumar 2004, Selvakumar 2006). Re-staining seems to increase the detection in sputum specimens transported in CPC solution (Selvakumar 2005). In addition, CPC inhibits mycobacterial growth, especially when inoculated in culture media including Middlebrook 7H9 and 7H10, which have an insufficient neutralizing activity for this quaternary ammonium compound. Therefore, specimens treated with CPC should be preferentially inoculated in egg-based media (Smithwick 1975). In a comparative study, sodium carbonate was found to be a better preservative of sputum specimens for AFB smear microscopy as well as culture (Bobadilla 2003). However, no comparative study has been undertaken to confirm this observation.



Figure 12-1: Example of packaging of infectious specimens.

12.2.3. Biosafety

This subject is thoroughly discussed in Chapter 11. Thus, in this chapter, laboratory safety will only be briefly addressed. Laboratory diagnosis of TB involves a risk of infection for laboratory personnel. Specimens with high mycobacterial loads, such as sputum or cultures, are often manipulated with limited biosafety measures, especially in low-resource countries. Although only limited information can be found in the literature on the risk of developing TB in laboratory personnel (Reid 1957, Kim 2007), studies in healthcare workers in contact with TB patients clearly show that TB can be considered as an occupational disease (Joshi 2006, Seidler 2005, Kilinc 2002).

Biosafety cabinets are seldom available in developing countries, and safety facilities for working with infectious specimens are limited. The only measure often taught to give any protection against infection with *M. tuberculosis* is processing infectious specimens behind the flame of a Bunsen burner, that is, the specimens are handled and the smear is prepared with the flame of the Bunsen burner interposed between the operator and the specimen. As far as we know, however, this measure has never been evaluated. Good laboratory practice is required for the protection of laboratory staff from infectious airborne bacilli, i.e. good ventilation, use of laboratory coats, surgical gloves and face masks, hand washing and regular disinfection of the laboratory floor and surfaces, especially benches, with a disinfectant that is active against mycobacteria. This disinfectant may be 70 % ethanol or sodium hypochlorite (house bleach) at a concentration of 0.2-0.5 % (see Chapter

11). Ultraviolet light, emitting rays of wavelength 254 nm, is very effective in killing the tubercle bacillus and other mycobacteria; it is also an additional measure for decontaminating work surfaces and killing airborne microorganisms (David 1973, Riley 1989). Good laboratory practice for handling TB specimens can be found in the guidelines by Kent (Kent 1985) and on the web page of the Centers for Disease Control and Prevention <http://www.cdc.gov/od/ohs/tb/tbdoc2.htm>; this web page also offers an inquiry for assessing safety in your laboratory at <http://www.phppo.cdc.gov/mpep/pdf/mtb/tb-ayl.pdf>.

12.3. Smear staining

12.3.1. AFB smear staining

AFB smear microscopy plays an important role in the early diagnosis of mycobacterial infections because most mycobacteria grow slowly and culture results become available only after weeks of incubation. In addition, AFB smear microscopy is often the only available diagnostic method in developing countries. Smear staining is based on the high lipid content of the cell wall of mycobacteria which makes them resistant to decolorization by acid-alcohol after the primary staining (see Chapter 3). To determine that a clinical specimen contains AFB, the specimen is spread onto a microscope slide, heat-fixed, stained with a primary staining, decolorized with acid-alcohol solution and counterstained with a contrasting dye in order to obtain better differentiation between the microorganism and the background. The slide is observed under the microscope for the detection of AFB. Several methods can be used for determining the acid-fast nature of an organism.

Two methods, Ziehl-Neelsen and Kinyoun, utilize basic fuchsin in ethanol for primary staining. In both cases, AFB appear red after decolorization with acid-alcohol. Ziehl-Neelsen is a hot acid-fast stain because the slide has to be heated during incubation with fuchsin. In contrast, Kinyoun staining is a cold acid-fast staining procedure and therefore does not require heating. Kinyoun's cold carbol fuchsin method is inferior to the Ziehl-Neelsen staining (Somoskovi 2001, Van Deun 2005). Details for the preparation of smear staining with the Ziehl-Neelsen method can be found in the following guidelines available on the internet: The WHO Laboratory Services in TB Control Part II Microscopy <http://www.phppo.cdc.gov/dls/ila/documents/lstc2.pdf>), the CDC Acid-Fast Direct Smear Microscopy Participant Manual (<http://www.phppo.cdc.gov/dls/ila/acidfasttraining/participants.aspx>), and the IUATLD technical guide (http://www.iuatld.org/pdf/en/guides_publications/microscopy_guide.pdf).

In the fluorochrome procedure, primary staining is done with auramine O. The AFB fluoresce yellow against a counterstain of potassium permanganate when observed with a fluorescence microscope. While the reading of fuchsin-stained smears requires 1000x magnification, fluorochrome-stained smears are examined at 250x or 450x. The lower magnification used in this staining method allows the microscopist to observe a much larger area of the smear during the same period of time and thus, fewer fields must be read. This makes the method faster and reduces laboratorist fatigue. Allegedly, fluorescent staining is more sensitive than Ziehl-Neelsen staining (Steingart 2006). However, it has been claimed that both methods have comparable sensitivity, provided procedural standards are followed, and a minimum of 300 fields are read with the Ziehl-Neelsen staining (Somoskovi 2001). Because of the rapidity of the fluorochrome method, laboratories processing large numbers of specimens should adopt this technique. A real disadvantage of the fluorochrome method is that fluorescence fades with time. For this reason, the slides must be read within 24 hours. This staining method is not often available in developing countries due to the high cost of the fluorescence microscope and, especially, that of its maintenance.

The results of the smear microscopy should be reported according to an internationally agreed quantitation scale.

Table 12-1: Quantitation scale recommended by the World Health Organization and the International Union Against Tuberculosis and Lung Disease

| Count on Ziehl-Neelsen /Kinyoun stain (1000x) | Count on Auramine/rhodamine (450x) | Report |
|---|------------------------------------|------------------|
| 0 | 0 | Non AFB observed |
| 1-9/100 fields | | Exact count |
| 10-99/100 fields | Observed count divided by 4 | 1+ |
| 1-10/field | | 2+ |
| > 10/field | | 3+ |

12.3.2. Quality control of AFB smear microscopy

Patients with infectious TB may remain undetected due to unreliable laboratory diagnosis. In addition, errors in AFB smear microscopy reading can result in patients being put on treatment without having the disease. Therefore, quality assurance of AFB sputum smear microscopy is essential, and the quality of laboratory

services should be considered a high priority of the National TB Control Programmes.

More details for quality assessment for AFB smear microscopy can be found in the following guidelines available on the internet: Quality Assurance of Sputum Microscopy

(<http://www.phppo.cdc.gov/dls/ila/acidfasttraining/participants/pdf/ParticipantModule10.pdf>) and Quality Assurance of Sputum Microscopy in the DOTS Programmes (http://www.wpro.who.int/NR/ronlyres/769B76D6-270F-4438-A4BE-D6B9FAC3902E/0/Quality_assurance_for_sputum_WP.pdf). These quality assurance programs are based on systematic monitoring of working practices, technical procedures, equipment and materials, including quality of stains, site evaluation of laboratory/quality improvement and also training, when needed.

An aspect usually overlooked in TB case finding and laboratory quality assessment is the quality of the sputum specimen itself. When the patient is thoroughly instructed on sputum sampling, the microscopic diagnosis of TB improves substantially (Alisjahbana 2005). Poor quality of the sputum specimen often results in AFB smear microscopy negative results (Hirooka 2004). Registering the quality of the sputum specimens received at the laboratory could help to improve sputum sampling. Satisfactory quality implies the presence of mucoid or mucopurulent material and a volume of 3-5 ml, although smaller volumes are acceptable if the consistency is adequate. If a relatively high percentage of the specimens received are saliva, the laboratory should report this to the medical staff, and instructions should be given to nurses and physicians on how to improve the quality of sputum sampling.

12.3.3. Concentrated sputum smears

Sputum is the most common specimen received for TB diagnosis. The minimum number of bacilli needed to detect their presence in stained smears has been estimated to be 5,000-10,000 per mL of sputum. For diagnosis, the sensitivity of AFB smear staining relative to culture has been estimated to vary from 50 % to over 80 %. Several studies have been published on improving smear microscopy performance using methods that concentrate the bacilli present in the sputum specimen. The methods consist of submitting the specimen to a liquefaction step prior to concentrating it by sedimentation or centrifugation. The smears are then performed from the sediment and stained for microscopic examination. The chemical method used for the liquefaction depends on the next step following concentration; smear staining only or smear staining followed by culturing.

The best known concentration procedure is the 'bleach microscopy method', in which the sputum is liquefied with sodium hypochlorite (NaOCl or household bleach), and concentrated by centrifugation before AFB staining. This technique is inexpensive and easy to perform. In addition, NaOCl is a potent disinfectant that also kills mycobacteria, thus reducing the risk of laboratory-acquired infection but, at the same time, rendering the method unsuitable for culturing. A significant improvement in the proportion of positive AFB smear results has been reported, ranging from 7 % to 253 % (Angeby 2004).

Other concentration methods should be used if the specimen is to be cultured. The sediment of a sputum specimen liquefied and decontaminated with sodium hydroxide-N-acetyl-L-cysteine method (see decontamination procedures below) and concentrated by centrifugation can also be examined by AFB smear staining. An advantage of this method is that the same sediment can be cultured, in contrast to those liquefied with the NaOCl method described above. Other methods involving sputum liquefaction with different substances, and concentration either by sedimentation or centrifugation, have been proposed. The methods using dithiothreitol (Murray 2003), chitin (Farnia 2004) and C(18)-carboxypropylbetaine (Scott 2002) have been evaluated favorably for the preparation of concentrated smears. Sputum concentration methods were recently reviewed (Steingart 2006) and compared with direct smears. The authors concluded that concentration by centrifugation and sedimentation with any of several chemical methods (including bleach) is more sensitive than direct AFB sputum smear examination.

12.4. Adenosine deaminase activity

Adenosine deaminase, also known as ADA, is an enzyme involved in the metabolism of purines. Its presence is needed for the breakdown of adenosine from food and for the turnover of nucleic acids in tissues. Many articles and reviews have reported the utility of ADA determination in body fluids (spinal, pleural, ascitic, pericardial) for the diagnosis of tuberculous meningitis (Kashyap 2006, Lopez-Cortes 1995), tuberculous pleurisy (Banales 1991, Goto 2003, Perez-Rodriguez 2000), peritoneal TB (Aston 1997, Gilroy 2006) and pericardial TB (Tuon 2006, Reuter 2006). AFB may be difficult to isolate from these specimens because they are often diluted in large fluid volumes. Biopsy specimens have a better yield, but biopsy is not always available in low-resource settings. ADA determination is simple and cheap and also has a high positive predictive value, especially in high endemic countries. The routine use of this method is justified in exudates of pleural, peritoneal and pericardial fluids. The specificity is very high in fluids with a

lymphocyte-to-neutrophil ratio higher than 0.75 (Burgess 1996, Diacon 2003). The suggested laboratory cut-off of ADA activity is 40 U/L for pleural, peritoneal and pericardial fluids and 10 U/L for cerebrospinal fluid. However different laboratories have established different cut-offs varying between 33 and 50 U/L.

As the description of the ADA assay is not readily available in the literature, it is detailed here. ADA is determined colorimetrically (Giusti 1974). A 25 μ L specimen is incubated for 60 min at 37°C with 500 μ L of 21 mM adenosine in 50 mM phosphate buffer pH 7.0. The reaction is interrupted by incubation with 1.5 mL of phenol nitroprusside at 37°C for 30 min (106 mM phenol, 0.17 mM sodium nitroprusside) in the presence of 1.5 mL of sodium hypochlorite (11 mM NaOCl plus 125 mM NaOH). The amount of ammonium ion released by the ADA reaction is determined as absorbance (optical density, OD) at 628 nm wavelength in a spectrophotometer (blue color). To control for the ammonium present in the patient's specimen before addition of exogenous adenosine, specimens without substrate are run in parallel (specimen blank). A standard (15 mM ammonium sulfate stock solution) and a reagent blank (50 mM phosphate buffer pH 7.0) are also included in the assay. The activity in the patient's specimen is calculated with the formula:

Activity in specimen = (OD specimen – OD specimen blank) / (OD standard – OD reagent blank) x 50 with the result expressed in Units/L.

12.5. Culture

12.5.1. Sterile or contaminated specimens

Acid-fast microscopy is easy and quick, but it does not confirm TB diagnosis because mycobacteria other than *M. tuberculosis* are also AFB in the smear microscopic examination. In addition, a high bacterial load is needed in the specimen to render an AFB microscopy result positive. Culture techniques have been estimated to detect as many as 10–1,000 viable mycobacteria per mL of specimen. Therefore, although most TB control programs do not support its widespread use due to enhanced laboratory complexity, biohazard and cost, in our view, all specimens should be submitted to culture, regardless of AFB smear microscopy results. A positive culture for *M. tuberculosis* confirms the diagnosis of active disease.

For culturing of mycobacteria, two types of clinical specimens are considered: contaminated specimens and specimens collected aseptically from normally sterile sites. Sterile specimens can be inoculated directly onto the culture medium. Specimens from non-sterile bodily sites are considered contaminated and therefore require processing before culturing in order to eliminate the associated flora. If not

properly eliminated, this flora will overgrow the culture medium long before mycobacteria have the chance to develop visible colonies.

12.5.2 Decontamination procedures

Several methods have been used to minimize culture contamination when specimens from non-sterile body sites are processed. Most of these methods include the digestion of mucus or organic debris and treatment to eliminate micro-organisms from the normal flora. Both steps are done to maximize the probability of isolating mycobacteria in culture. No single decontamination method is applicable to all circumstances, laboratories and clinical specimens; therefore, a laboratory should use the best suited method that keeps the contamination rate between 3 % and 5 %. A contamination rate lower than 3 % may indicate that the procedure used is too harsh and may be killing the mycobacteria (Della Latta 2004).

The decontamination methods most commonly used are (Table 12-2):

- **Sodium hydroxide.** This method uses sodium hydroxide at concentrations ranging between 2 % and 4 % to digest and, at the same time, decontaminate the specimen. Each laboratory should determine the lowest concentration for optimal digestion and decontamination (Della Latta 2004).
- **N-acetylcysteine-sodium hydroxide, sodium chloride and sodium hydroxide.** This method, one of the most used worldwide, uses N-acetylcysteine for mucus digestion and sodium hydroxide as the decontaminant (Della Latta 2004).
- **Oxalic acid.** This method is recommended for decontamination of clinical specimens that may have *Pseudomonas aeruginosa* as a contaminant, usually urine specimens and pulmonary specimens from cystic fibrosis patients (Della Latta 2004, Cooper 1930).
- **Ogawa-Kudoh.** This is a very simple and practical decontamination method that obviates the use of specimen centrifugation prior to culturing. The procedure was described by Kudoh using sodium hydroxide as the digestant-decontaminant and inoculation in a modified Ogawa media (Kudoh 1974). It does not require laboratory facilities and can be performed in the field, as described below:

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- With the aid of a sterile cotton swab, obtain a significant portion of the specimen
- Submerge the applicator in a tube containing 3 mL of 4 % sodium hydroxide solution
- Incubate at room temperature for two minutes
- Remove the applicator from the sodium hydroxide solution
- Inoculate a tube containing modified Ogawa culture medium
- With the same applicator, make a smear for AFB microscopic examination

Table 12-2: Decontamination methods

| Method | Use | Advantage | Disadvantage |
|---|--|---|--|
| Sodium hydroxide | Laboratories using concentration by centrifugation | - Digestion / decontamination at the same time when used at a final concentration of 2 % - Low cost | - Precise timing needed to avoid killing mycobacteria - may kill some mycobacteria even at 2 % concentration |
| N-acetylcysteine - sodium chloride - sodium hydroxide | - Most used in developed countries - Used in combination with centrifugation | - Good mucolytic action - Use of NaCl as mucolytic reduces NaOH concentration and its potential deleterious action on mycobacteria | - Short shelf-life of prepared reagents (24 h) - Higher cost |
| Oxalic acid | - Recommended to eliminate <i>P. aeruginosa</i> contamination (e.g. in urine, specimens from cystic fibrosis patients) | - Effective in inhibiting overgrowth by <i>Pseudomonas</i> | - Use restricted to inhibit <i>Pseudomonas</i> |
| Ogawa-Kudoh | - Ideal method for low-resource settings | - Centrifugation or concentration is not necessary - Low cost - Can be used in the field | - May have higher contamination rates |
| Cetyl pyridinium - sodium chloride | - For preservation and digestion / decontamination while in transport to the laboratory | - Avoids overgrowth of contaminants for up to eight days | - Egg-based media required since compound remains active in agar and may be deleterious to mycobacteria |

- **Cethyl pyridinium and sodium chloride.** This method is useful to preserve specimens from contaminant flora overgrowth while in transit to the laboratory and also fulfills the decontamination step required prior to culture. It has demonstrated viability of mycobacteria exposed for eight days to CPC (Smithwick 1975).

12.5.3. Concentration of mycobacteria

With the exception of the Ogawa-Kudoh method, specimens are inoculated after a concentration step, which is done by spontaneous sedimentation or, more frequently, by centrifugation. Many laboratories report centrifugation speeds in revolutions per minute (rpm). However, this speed measure is related to each particular centrifuge. The measure of sedimentation efficiency is the relative centrifugal force (RCF), which considers the radius from the centre of the rotating head to the bottom of the centrifuge tube (R max) and the centrifuge speed (rpm). The RCF may be calculated from the formula:

$$\text{RCF} = 1.12 \times \text{R max (in mm)} \times (\text{rpm}/1,000) \times 2$$

The optimum RCF and centrifugation time combination have been determined to be 4,000 x g for 15 min. (Perera 1999) but an RCF of 3,000 x g applied for 15 min, or an RCF of about 2,000 to 2,500 x g applied for 20 min, is still considered adequate to concentrate mycobacteria in clinical specimens. Lower RCF and/or centrifugation times may lead to a considerable loss of mycobacteria. The sediment of the concentration step will be inoculated directly onto the culture medium, or after decontamination in the case of non-sterile specimens that did not undergo decontamination before the concentration step.

12.5.4. Culture media

Different culture media are in use for the isolation of mycobacteria. The most common are based on egg and also contain high concentrations of malachite green to overcome contamination with other bacteria. Detailed guidelines for the preparation of the most widely used egg-based media, Löwenstein-Jensen and Ogawa, are freely accessible on the internet (Laboratory Services in Tuberculosis Control Part III: Culture: <http://www.phppo.cdc.gov/dls/ila/documents/lstc3.pdf>). In general, after the centrifugation step, sediments are inoculated onto two Löwenstein-Jensen slants. In areas with a high incidence of bovine TB, a tube with Stonebrink

medium should be added. *M. bovis* and other species of the *M. tuberculosis* complex (*M. microti* and *M. africanum*) are unable to use glycerol as a carbon source due to the lack of a functioning pyruvate kinase. Thus, these organisms will often fail to grow on Löwenstein-Jensen medium, which contains glycerol as the only available carbon source (Keating 2005, see Chapter 3). Stonebrink medium has the same composition as Löwenstein-Jensen, with the exception that glycerol is replaced by 0.5 % sodium pyruvate. Many diagnostic laboratories that employ egg-based medium for the isolation of mycobacteria, omit the use of Stonebrink medium. This probably leads to an underestimation of the actual weight of *M. bovis* as a human TB agent, especially in developing countries (see Chapter 8).

The Ogawa medium is another egg-based medium, which is comparable in its composition with Löwenstein-Jensen. It is more economic because it replaces asparagine by sodium glutamate, an amino acid more readily available and much cheaper. Modified Ogawa medium (pH 6.4) is the same egg-based Ogawa medium that has been acidified in such a way as to allow the direct inoculation of specimens decontaminated by the Kudoh method. This combination is very suitable for culturing sputum specimens in rural settings.

Middlebrook 7H10 and 7H11 are agar-based media. Their basic ingredients are commercially available: powder base, agar and Middlebrook OADC enrichment. Middlebrook 7H9 is a liquid medium and may be prepared from commercially available powder base supplemented with Middlebrook ADC enrichment after sterilization. Incubation in a 5 % to 10 % CO₂ atmosphere is recommended. Middlebrook media have been shown to achieve slightly higher isolation yields than egg-based media, but are considerably more expensive.

Details for the preparation of the above mentioned media can be found in the annual on Laboratory Services in Tuberculosis Control Part III: Culture, which is freely available on the internet at <http://www.phppo.cdc.gov/dls/ila/documents/lstc3.pdf>.

The BACTEC TB-460 system was the first semi-automated system to appear on the market for mycobacteria culturing, and still serves as the bench-mark for quality, reliability and performance. In the last decade, several new commercial culture media have been introduced, such as the Mycobacteria Growth Indicator Tube (MGIT), Bract/Alert, ESP Mice, MB Redox and KRD "Niche B", biphasic Septic-Check AFB and Mice-Acid, and BACTEC MGIT960 systems. Like BACTEC TB-460, these newer systems are based on liquid media. Liquid culture media has been proven to be significantly more sensitive than egg-based solid media for the isolation of mycobacteria from clinical specimens (Hines 2006). However, one disad-

vantage is the much higher price, which is often too high for cost-effective TB diagnosis in resource-limited countries. These methods are described in detail in Chapter 14.

Blood agar is an alternative culture medium for isolation of mycobacteria. Historically, microbiologists and medical students have been taught for decades that isolation of mycobacteria requires a defined, egg-based medium such as Löwenstein-Jensen. In fact, the tubercle bacillus does not have special growth requirements (see Chapter 3), and blood agar is at least as efficient as the widely recommended egg-based media. *M. tuberculosis* grows within one to two weeks on blood agar plates, and it has been reported that the average number of colonies isolated from clinical specimens on blood agar is significantly higher than the number of colonies on the egg-based medium (Dracut 2003). Preliminary studies suggested that blood agar can also be used as an alternative medium for susceptibility testing of *M. tuberculosis* against isoniazid, rifampicin, streptomycin and ethambutol. Reportedly, results are obtained much earlier with blood agar (2 weeks) than with 7H10 agar (3 weeks) (Coban 2005, Coban 2006). Desiccation is prevented by sealing the plates with tape or by using tubes instead of plates. Blood agar plates are readily available in most laboratories dedicated to general bacteriology, and thus, in the absence of more specific media, they could be used for the culture of mycobacteria, especially in resource-limited countries.

12.5.5. Reading of results

Conventional culture media such as those based on egg and agar should be examined for growth twice a week for the first four weeks starting on day 3 to 5 post-inoculation, and thereafter, once a week until the eighth week. All specimens showing growth in culture should be confirmed as AFB by smear microscopy of the colonies and reported immediately as “culture positive for mycobacteria pending identification”. All cultures reported positive for mycobacteria should be identified to the level of species using either biochemical or molecular methods.

M. tuberculosis bacilli are slow-growing mycobacteria. This means that, in primary isolation, they hardly show any visible growth during the first week of culture. On egg-based media they produce characteristic non-pigmented colonies, with a general rough and dry appearance simulating breadcrumbs (Figure 12-2). On agar-based media, the colonies appear flat, dry and rough with irregular edges.



Figure 12-2: Colonies of *Mycobacterium tuberculosis* in Löwenstein Jensen medium (Courtesy of A. Martin).

Cross-contamination can produce false positive results. The causes of false-positive cultures include contamination of clinical devices (i.e. bronchoscopes), clerical errors, and laboratory cross-contamination. In a recent review, false-positive cultures were identified in 13 of the 14 studies that evaluated 100 or more patients; the median false-positive rate was 3.1 % (interquartile range, 2.2 % - 10.5 %). Patients having only one positive culture when two tubes were inoculated and patients with only a few colonies in the culture should be further evaluated for the possibility of a false-positive result (Burman 2000).

12.6. Identification

12.6.1. Biochemical procedures

The final species identification of *M. tuberculosis* is based on characteristics such as slow growth, colony morphology, and biochemical tests. From a practical point of view, most isolates from human disease belong to the species *M. tuberculosis*. However, depending on geographical and epidemiological circumstances, it may be necessary to differentiate species within the *M. tuberculosis* complex (see Chapter 8).

An initial identification as *M. tuberculosis* is defined on AFB bacilli from slow-growing, non-pigmented colonies that are niacin positive, are inhibited by p-nitrobenzoic acid and display nitratase activity. Additional tests that confirm an

isolate as *M. tuberculosis* are susceptibility to pyrazinamide, growth on thiophene carboxylic acid hydrazide, absence of catalase production at 68°C and absence of iron uptake. Table 12-3 summarizes the differential characteristics of species in the *M. tuberculosis* complex.

Table 12-3: Colony morphological and biochemical characteristics of species in the *M. tuberculosis* complex

| Test | <i>M. tuberculosis</i> | <i>M. bovis</i> | <i>M. bovis</i> BCG | <i>M. africanum</i> | <i>M. microti</i> | " <i>M. canettii</i> " |
|--|------------------------|------------------|------------------------|---------------------|-------------------|------------------------|
| Morphology | rough | rough | rough | rough | rough | smooth |
| Pyruvate rather than glycerol as carbon source | - | + | + | - | - | - |
| Pyrazinamidase | + | - | - | + | + | + |
| Niacin | + | - | - | +/- | + | - |
| Nitratase | + | - | - | +/- | - | + |
| Urease | +/- | - | + | +/- | +/- | + |
| Susceptibility to TCH | R | S | S | S | S | R |
| O ₂ requirement | aerobic | Micro-aerophilic | aerobic | Micro-aerophilic | Micro-aerophilic | Unknown |

R= resistant, S= susceptible, TCH= Thiophene-2-carboxylic acid hydrazide

12.6.2. Main biochemical tests to identify *M. tuberculosis*

- **Niacin accumulation test.** Nicotinic acid or niacin is produced by all mycobacteria, but some species, such as *M. tuberculosis*, *Mycobacterium simiae* and *M. bovis* BCG, excrete it due to a blockade in their scavenging pathway. The excreted niacin accumulates in the culture medium and is evidenced in the presence of cyanogen halide with a primary amine (Figure 12-3). Niacin-negative *M. tuberculosis* strains are extremely rare.
- **Growth in the presence of p-nitrobenzoic acid.** This compound inhibits the growth of several species in the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* (Tsukamura 1984, Leão 2004).
- **Nitrate reduction test.** This test is particularly useful for differentiating *M. tuberculosis*, which gives a positive reaction, from *M. bovis*, which is negative (Tsukamura 1984, Vincent 2003).

- **Catalase test.** Catalase is an intracellular enzyme that transforms hydrogen peroxide to oxygen and water. The 68°C catalase is a heat-tolerance test measuring the catalase activity at high temperature. Characteristically, *M. tuberculosis* gives negative results, as do other species in the *M. tuberculosis* complex. (Vincent 2003).
- **Pyrazinamidase test.** Pyrazinamidase is an enzyme that hydrolyzes pyrazinamide to ammonia and pyrazinoic acid. The test is useful to differentiate *M. tuberculosis* (positive) from the other species of the *M. tuberculosis* complex (negative), with the exception of “*M. canettii*”, which is also positive. Some strains of *M. tuberculosis* may acquire resistance to pyrazinamide due to selective pressure induced by treatment with this drug. These strains give a negative pyrazinamidase test (unable to transform pyrazinamide to pyrazinoic acid, the active form of the drug) (Vincent 2003).
- **Growth in the presence of thiophen-2-carboxylic acid hydrazide.** This test is useful to distinguish *M. tuberculosis*, which grows in the presence of this compound, from other members of the *M. tuberculosis* complex. “*M. canettii*” and most non-tuberculous mycobacterial species are also positive to this test (Vincent 2003, Leão 2004).



Figure 12-3: Niacin test (Courtesy of A. Martin).

12.6.3. Isolation of non-tuberculous mycobacteria

Non-tuberculous mycobacteria (NTM) are ubiquitous organisms that are frequently isolated from environmental sources, including surface water, tap water, and soil. Specimens from the respiratory tract often grow NTM. The isolation of NTM species from a respiratory specimen is not enough evidence for the presence of NTM lung disease, the diagnosis of which must rely on clinical, radiographic, and bacteriologic criteria. Both the American Thoracic Society (<http://www.thoracic.org/sections/publications/statements/pages/mtpi/nontuberc1-27.html>) and the British Thoracic Society (<http://www.brit-thoracic.org.uk/c2/uploads/OppMyco.pdf>) have issued guidelines regarding the diagnosis of lung disease due to NTM. Both guidelines highlight the difficulty inherent in differentiating patients with clinical lung disease caused by NTM from those in whom the isolation of clinical specimens raises a suspicion of disease. At least three respiratory specimens should be evaluated from each patient to establish the clinical significance of the infection. The NTM species most frequently associated with pulmonary disease are *M. avium*, *Mycobacterium kansasii* and *Mycobacterium abscessus*.

Wound infections, prosthetic valve endocarditis, infections complicating mammary augmentation surgery, and other cutaneous/subcutaneous infections have been attributed to rapidly growing mycobacteria. *Mycobacterium fortuitum*, *M. abscessus*, and *Mycobacterium chelonae* are the most common mycobacteria implicated in these infections, which are thought to be caused by local environmental strains or contaminated commercial surgical materials, devices or solutions for injection. Rapidly growing mycobacteria often grow on classical bacterial culture media, especially on blood agar plates. However, due to their delay in forming visible colonies (up to 10 days), they are usually not detected in the routine bacteriology laboratory. They can also be isolated on most media available for the isolation of mycobacteria. Although the optimum temperature for most species is 30-32°C, they also grow at 36°C to 37°C, the standard temperature for isolation of the tubercle bacillus. Mycobacteria should be identified at the species level before starting treatment, because different species display different antibiotic resistance patterns. An extensive review on rapidly-growing NTM has been recently published (Brown-Elliott 2002).

Certain NTM are fastidious and special culture conditions or growth requirements should be observed for their isolation. Because of this, the organisms are not be isolated using routine mycobacterial culture techniques. If disease by one of these

mycobacteria is suspected, the bacteriologist must be notified so that appropriate cultivation conditions can be implemented. *Mycobacterium marinum* causes localized cutaneous lesions in patients with a history of a penetrating cutaneous injury and prolonged or repeated aquatic exposure. This microorganism grows at 30°C and its growth is inhibited at 37°C. Two other mycobacterial pathogens require special conditions for laboratory culture: *Mycobacterium haemophilum*, which causes cutaneous, joint, or pulmonary infection in immunocompromised patients and lymphadenitis in children, grows preferentially at 30°C to 32°C, and requires iron-supplemented media i.e. egg-based medium supplemented with iron complexes or blood agar medium; and *Mycobacterium genavense*, which produces disseminated infections in HIV/AIDS patients, grows in Middlebrook 7H11 agar supplemented with mycobactin J, and requires prolonged incubation periods (Coley 1992).

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